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Studies on Redox-proteins and Cytokines in Inflammation and Cancer

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Cover picture: Co-clustering of PDI and TNF-receptor

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This thesis is dedicated to

my beloved Father and Mother

Abstract

The redox state in the cell plays a major role in determining vital functions and its major imbalance can lead to severe cell injury or death. Redox active proteins and cytokines involved in this process includes thioredoxin (Trx), protein disulfide isomerase (PDI), and tumor necrosis factor (TNF) superfamilies. Trx is a multipotent protein and key regulator of cellular redox balance operating in synergy with Trx reductase and NADPH (the Trx system). Trx has gene regulatory activity of several transcription factors. It also controls in a fascinating way redox-sensitive "on-off" decisions for apoptotic or hypertrophic pathways. Trx protects against H_2O_2 and TNF-mediated cytotoxicity, a pathway in which TNF receptor-binding generates ROS. TNF is an autocrine growth factor and survival factor *in vitro* and *in vivo* for B-type of chronic lymphocytic leukemia (B-CLL) cells. The overall aim of this study was to investigate the importance of redox active proteins and cytokines in inflammation and cancer. We focused on: i) the role of Trx, TrxR, and selenium in carcinogenesis and in resistant cancer cells. ii) the importance of Trx in cancer cells and the redox regulation of TNF and its receptors TNFR1 and TNFR2. iii) the potential role of Trx as a key regulator in cellular redox balance, in the pathogenesis of cardiac dysfunction; its relationship to stress response parameters. iv) whether unmutated CLL (U-CLL) responses to PKC and ROS pathways were different from mutated CLL (M-CLL) responses.

Our results demonstrate pronounced selective selenium-mediated apoptosis in therapy resistant cells and suggest that redox regulation through the Trx system is an important target for cancer therapy. Trx was strikingly elevated in heart failure cases compared with controls signifying an adaptive stress response that is higher the more severe the disease. TNF autocrine release was redox modulated and the TNF receptors interacted at the cell surface membrane with the redox-active PDI, which excerted a stringent redox-control of the TNFR signaling. The proliferative response as well as increase of autocrine TNF and Trx were higher in U-CLL than in M-CLL.

The overall conclusion of the four papers included in this thesis is that redox-active proteins and cytokines plays an important role in control and regulation of cancer and inflammation. Furthermore, redox regulation via thioredoxin by selenium, may offer novel treatment possibilities for resistant tumors disease.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Våra celler har en förmåga att bibehålla en inre reducerande miljö trots en stark oxiderande omgivning. Redoxsignaler utgör en länk mellan interna och externa stimuli. Det har under de senaste åren klartlagts att redox-reglering är en viktig funktion i ett flertal biologiska förlopp såsom DNA-syntes, enzymaktivering, selektivt genuttryck, och cellcykel reglering. Cellens redox-status kan fluktuera mellan ett mer oxiderat och ett mer reducerat tillstånd, vilket i sin tur indikerar att det finns reglermekanismer. Aktiva redox-proteiner och cytokiner såsom tioredoxin (Trx), tumör nekros fakor (TNF) och protein-disulfid isomeras (PDI) har viktiga funktioner för att kontrollera en sund redoxbalans.
Trx återfinns i både prokaryoter till eukaryoter och spelar en nyckelroll. Trx förekommer i skilda biologiska förlopp såsom celltillväxt. Trx systemet utgörs av Trx och flavoenzymet tioredoxin-reduktas (TrxR) samt NADPH; tillsammans bildar de ett potent protein-disulfid reduktas system. Trx är också viktigt för kontroll av cancer, inflammatoriska och immunologiska svar. Hos cancerceller kan man ofta se störningar i den normala interaktionen mellan dessa cytokiner, i samspelet med andra celler genom adhesionsmolekyler, eller vid cell-matrix interaktioner, med följden att cellerna delar sig vid fel tidpunkt och på fel plats. Störningarna beror på att cancercellen, som har sitt ursprung i den normala cellen, genom en serie ofta diskreta förändringar i arvmassan, uppnått ett tillstånd med ohämmad celldelningar.

Vi har tittat på i) vilken roll Trx, TrxR och selen har och hur dessa proteiner påverkar cancerceller och cytostatikaresistenta celler. ii) vilken roll Trx och TNF har vid kontroll av redoxbalans vid stress, cancer och kronisk hjärt sjukdom. iii) hur muterade och omuterade kronisk lymfatiska leukemi (KLL) celler skiljer sig med avseende på redoxreaktioner efter påslagna PKC-signaleringsvägar.

Vi har observerat att selen orsakar att cancerceller dör en normal död (apoptos) och våra resultat indikerar att selen möjligen kan användas för cancerbehandling. Vi har också erhållit resultat som visar att Trx modulerar och inducerar frisättning av TNF. Vår grupp rapporterade att Trx förlängde överlevnaden på cancercellerna. TNF-receptor interaktionen med PDI kontrollerar TNFR signalvägen visades i ett delarbete.

Slutligen visar våra resultat att redoxaktiva proteiner och cytokiner har en nyckelroll för att kontrollera redoxbalansen i celler. Dessutom torde selen kunna användas för behandling av resistenta cancerceller i framtiden.

List of Publications

This thesis is based on the following original scientific papers, which will be referred to in the text by the Roman numerals:

I. Kerstin Jönsson-Videsäter, Linda Björkhem-Bergman, **Akter Hossain**, Anita Söderberg, Lennart C. Eriksson, Christer Paul, Anders Rosén, Mikael Björnstedt. (2004)."Selenite-induced apoptosis in doxorubicin-resistant cells and effects on the thioredoxin system." *Biochem Pharmacol* **67***: 513-22.*

II. Andreas Jekell*, **Akter Hossain***, Urban Alehagen, Ulf Dahlström, Anders Rosén. (2004). "Elevated circulating levels of thioredoxin and stress in chronic heart failure." *Eur J Heart Fail 6: 883-90.* (*Shared first authorship)

III. **Akter Hossain**, Anita Söderberg, and Anders Rosén. Membrane protein disulfide isomerase regulates TNF-receptors 1 and 2 via direct molecular interaction: Redox-control of TNF autocrine loop in CLL. (manuscript)

IV. **Akter Hossain**, Ann-Charlotte Bergh, Mats Linderholm, Anders Rosén, and Eva Bäckman. Increased thioredoxin and TNF expression in unmutated CLL compared with mutated CLL cells after protein kinase C activation. (manuscript)

Abbreviations

ADF	adult T-cell leukemia-derived factor
AIF	apoptosis-inducing factor
ALS	amyotrophic lateral sclerosis
AP-1	activator protein-1
Apaf-1	apoptotic protease activating factor-1
ASK-1	apoptosis signal-regulating kinase-1
ATF	activating transcription factor
ATL	adult T-cell leukemia
ATP	adenosine triphosphate
B-CLL	B-cell chronic lymphocytic leukemia
BZIP	basic leucine zipper
Ca^{2+}	calcium
CD	Crohn´s disease
CHF	chronic heart failure
DHLA	dihydrolipoic acid
DISC	death inducing signaling complexs
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activating cell sorting (used as generic name flow cytometry and cell sorting)
FAD	flavin adenine dinucleotide
GPX	glutathione peroxide
$GS^{\cdot-}$	pro-oxidant glutathione radical
GSH	glutathione, reduced
GSSG	glutathione, oxidized
H_2O_2	hydrogen peroxide
HTLV	human T-cell lymphotropic virus
IBD	inflammatory bowel disease
IC_{50}	half maximal inhibitory concentration
IFN	interferon

IG	immunoglobulin
IGHV	immunoglobulin heavy chain variable region
IκB	inhibitory protein κB
IL	interleukin
JNK	Jun N-terminal kinase (also called SAPK)
kDa	kilodalton
LA	lipoic acid
LDL	low-density lipoprotein
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAP	mitogen-activated protein
MFI	mean fluorescence intensity
MPT	mitochondrial permeability transition
MS	multiple sclerosis
MW	molecular weight
Na_2SeO_3	sodium selenite
NAC	*N*-acetyl-L-cysteine
NADPH	nicotine adenine dinucleotide phosphate
NF-κB	nuclear factor-κB
NGFR	nerve growth factor receptor
NO	nitric oxide
NOX	NADPH-oxidase
p53	tumor suppressor protein 53
PBMC	peripheral blood mononuclear cells
PD	Parkinson's disease
PDI	protein disulfide isomerase
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
RA	rheumatoid arthritis
Redox	reduction/oxidation
RNA	ribonucleic acid
ROS	reactive oxygen species
Se	selenium

SeCys	selenocysteine
SeP	selenoprotein P
SH-	thiol
SOD	superoxide dismutase
TDs	transactivation domains
TMX2	thioredoxin-related transmembrane protein-2
TNF	tumor necrosis factor
TNFR	tumor necrosis factor-receptor
TRAF	TNF-receptor associated factor
TRANK	Trx peroxidase-related activator of NF-κB and c-Jun N terminal kinase
Trx	thioredoxin
Trx-$(SH)_2$	reduced thioredoxin
Trx-*l*	thioredoxin-like protein
TrxR	thioredoxin reductase
Trx-S_2	oxidized thioredoxin

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Oxidative stress and reactive oxygen species:

Oxidative stress is a term used to describe damage to animal or plant cells by reactive oxygen species (ROS). It is defined as an imbalance between free radicals and antioxidants. This imbalance can affect a specific molecule or the entire organism. The level of oxidative stress is determined by the balance between the rate at which oxidative damage is induced and the rate at which is efficiently repaired and removed. (Figure 1) Endogenous ROS which are products of normal and essential metabolic reactions including cellular respiration, react with nucleic acids, lipids, proteins and sugars. Exogenous ROS sources include environmental pollutants, sunlight, ionizing radiation, smoke, asbestosis.

Free radicals such as reactive oxygen species are atoms or groups of atoms with an unpaired number of electrons.[1] Examples of free radicals are hydrogen peroxide, hydroxyl radical, nitric oxide, peroxynitrite, singlet oxygen, superoxide anion and peroxyl radical.[2]

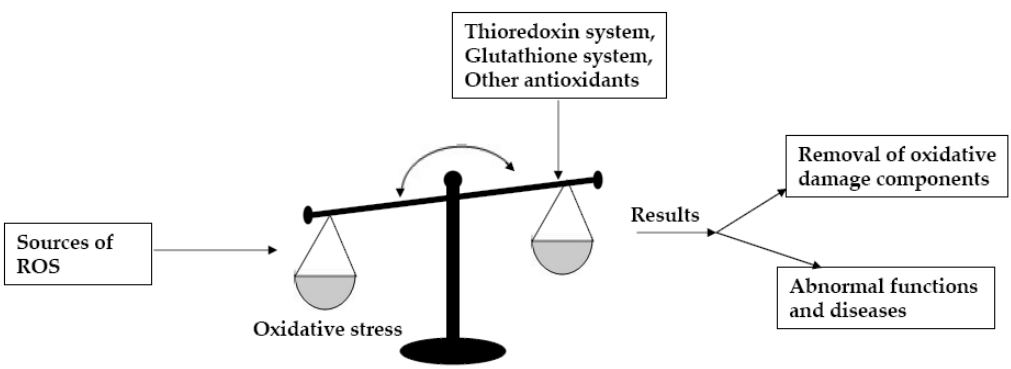

Figure 1. Simplified presentation of redox balance in cells.

Free radical formation is increased by immune cell activation, inflammation, ischemia, infection, cancer, and chronic heart disease. The radicals react with (oxidize) various cellular components including DNA, proteins, and lipid/fatty acids which leads to DNA damage, mitochondrial malfunction, cell membrane damage and eventually cell death (apoptosis).

ROS are essential:

ROS have a beneficial role in areas including intracellular signaling and redox regulation, kinase and phosphatase activity and gene expression via transcription factor like nuclear factor κB (NFκB) and activator protein-1 (AP-1).[3, 4, 5] For synthesis of thyroxine, hydrogen peroxide (H_2O_2) is required for the proper transfer of iodine.[6] In order to kill bacteria, macrophages and neutrophils generate ROS via NADPH-oxidases (NOX), called ROS-burst.[7]

Defense against ROS:

The antioxidant defense system protects against the harmful effects of ROS. It consists of two types, non-enzymatic system and enzymatic system, including low molecular weight compounds with antioxidant properties.

Non-enzymatic antioxidant system:

The non-enzymatic antioxidants include lipid soluble vitamins, vitamin E and vitamin A or provitamin A (beta-carotene), the water soluble vitamin C, glutathione (GSH), selenium, ubiquinone, and polyphenols.

Vitamins:

Vitamin C:

Water soluble vitamin C (or ascorbic acid) is capable of scavenging various ROS.[8] It´s deficiency leads to a well known disese called scurvy. Since ascorbate is water soluble, it works both inside and outside cells to prevent ROS accumulation. It donates electrons to free radicals such as hydroxyl

and superoxide radicals and quench their reactivity. The oxidized ascorbate called dehydroascorbate, is reduced by GSH or the thioredoxin (Trx) system. It also works in parallel with glutathione peroxidase and vitamin E. The combination of both vitamins prevent atherosclerotic progression in hypercholesterolemic persons.[9] and inhibit the early progression of coronary arteriosclerosis after heart transplantation.[10]

Vitamin E or α-tocopherol:
Vitamin E is a fat soluble vitamin, which is also known as an anti-sterility vitamin and a powerful antioxidant. It can break covalent links that ROS have formed between fatty acid side chains in membrane lipids and protect the cell against ROS. It inhances enthothelial cell function, traps oxygen free radicals[11] and inhibit monocyte endothelial adhesion and cytokine release.[12] α-Tocopherol is considered to be a scavenger for lipid peroxyl radical. Epidemiological studies suggest that vitamin E decrease the incidence of Alzheimer´s disease, Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis.[13]

Glutathione (GSH):
GSH is the smallest intracellular thiol (SH) molecule and it is synthesized in the body from the three amino acids cysteine, glutamine and glycine. It plays a principal role in the maintenance of the intracellular redox state. It is a nucleophilic scavenger and an electron donor via the sulfhydryl group. The reduced form of GSH is present in the cystol in millimolar concentrations. Pro-oxidant GSH radicals ($GS^{\cdot}$) react with another ($GS^{\cdot}$) and forms oxidized GSH (GSSG). It is reduced by GSH reductase. GSH reacts with reactive nitrogen species peroxynitrite to form s-nitrosoglutathione, which is cleaved by the Trx system to release GSH and NO.[14] GSH is able to reduce oxidized vitamin C and E back to their reduced state. It also act as a cofactor for the selenium–containing GSH peroxidases, which are major antioxidants.

Selenium:
Selenium is an essential biological trace mineral with both antioxidant and pro-oxidant effects. In low concentrations selenium protect cells against the effects of free radicals (antioxidant). Thus affecting oxidative stress, DNA repair, DNA methylation, inflammation, apoptosis, cell proliferation, carcinogen metabolism, hormone production and immune function.[15] In higher concentration (5-10 μM) selenium has pro-oxidant affects.

Ubiquinone (Q10):
Ubiquinone (Q10) is an important powerful lipid soluble antioxidant molecule consisting of a redox-active quinoid nucleus and a monosaturated tran-isoprenoid side chain.[16] It plays a role in the electron-transport chain to produce ATP, which is the major source of energy.

Polyphenols:
The main source of polyphenol antioxidants is nutrients, which can be found in our diet. For example, most legumes, fruits such as apples, blackberries, cherries, cranberries, grapes, pears, plums, raspberries, and strawberries; and vegetables such as broccoli, cabbage, celery, onion and parsley are rich in polyphenol antioxidants. Red wine, chocolate, green tea, olive oil, honey and many grains are alternative sources. Polyphenols can be divided into four subgroups bioflavonoids, anthocyanins, proanthocyanidins and xanthones. Bioflavonoids is an important subgroup that can prevent inflammatory effects in for example cardiovascular disease,[17] including downregulation of oxidative LDL.[18] It has been shown to reduce ROS levels *in vivo*.[19]

Lipoic acid (LA):
An alternative name for Lipoic acid is universal antioxidant due to it´s water and fat solubility. It can be found in spinach and liver but the body is also able to synthesize its own supply. LA is metabolized to its reduced form, dihydrolipoic acid (DHLA), by mitochondrial lipoamide dehydrogenase. LA and DHLA together form a redox couple.[20] Lipoic acid can be reduced by lipoamide dehydrogenase, GSH reductase and Thioredoxin reductase (TrxR) and the best reductant is TrxR.[21]

Enzymatic antioxidant system:

The enzymatic antioxidant system includes superoxide dismutase, catalase and peroxidases.

Superoxide dismutase (SOD):

SOD is an endogenously produced intracellular enzyme with an active centre occupied by copper (Cu), zinc (Zn), manganese (Mn) or iron (Fe).
i) Cu/Zn-SOD is localized in the cytoplasm and uses copper and zinc to maintain its catalytic activity and protect the cytoplasm. Cu/Zn-SOD is also secreted from the cells into the extracellular matrix (EC-SOD).[22]
ii) Mn-SOD resides in the mitochondria and protect the mitochondria from free radical damage.
iii) A third extracellular SOD has been described which contains Cu-SOD.
iv) Fe-SOD is found both in prokaryotes and in eukaryotes.[23] SOD neutralizes superoxide thus protecting cells from ROS damage.

$$2O_2^{\cdot -} + 2H^+ + SOD \longrightarrow H_2O_2 + O_2$$

Through a feedback system, the respective enzymes that interact with superoxide and H_2O_2 are tightly regulated.

Catalase:

Catalase is an antioxidant enzyme which works closely with SOD to prevent free radical damage to the body. It is mainly located in cellular peroxisomes but can also be detected in mitochondria.[24] Hydrogen peroxide is produced in the body in different ways, (a) When SOD converts the dangerous superoxide radical to hydrogen peroxide. b) When fatty acids are converted to energy (c) When white blood cells attack and kill bacteria.

Catalase converts hydrogen peroxide to harmless water and oxygen.

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + O_2$$

NADPH protect the catalase from oxidative damage.[25]

Glutathione-related enzymes:

Glutathione peroxide (GPx):

GPx is a selenium-dependent enzyme which reduces H_2O_2 (Figure 2) and other peroxides in presence of GSH as the source of electrons. There are at least four different GPx described in mammals.[26] The classical cellular form of GPx (cGPx) or Gpx1 and Gpx4 is dispersed throughout the cytoplasm, but GPx1 activity is also found in mitochondria. The extracellular form of GPx or GPx3 is genetically distinct from cellular GPx and has been detected in several tissues in contact with body fluids, i.e. in the kidney. It is structurally similar with GPx1.[27]

Gpx2 is found in the gastrointestinal tract, forming a barrier against hydroperoxides from the diet.[26] All GPx reduce hydrogen peroxide and alkyl hydroperoxides and utilize GSH as thiol substrate.

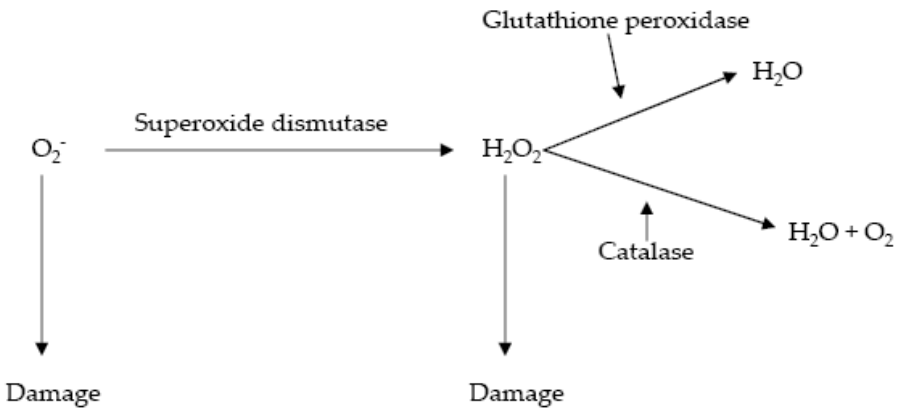

Figure 2. Detoxification of free radical by the antioxidant enzyme.

Selenium:

Selenium is a trace element (atomic weight 79) with the chemical symbol Se and can exsist as a gray crystal, red powder or vitreous black form.
In 1817 Jöns Jakob Berzelius a Swedish physician and scientist discovered selenium.[28] Selenium is a nonmetal and chemically related to sulfur and tellurium. The main inorganic dietary form of selenium is sodium selenite (Na_2SeO_3) and organic forms are selenomethionine and selenocysteine. (Figure 3) Selenium occurs in nature as six stable isotopes: ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{82}se and ^{80}Se. There are 24 others unstable selenium isotopes.

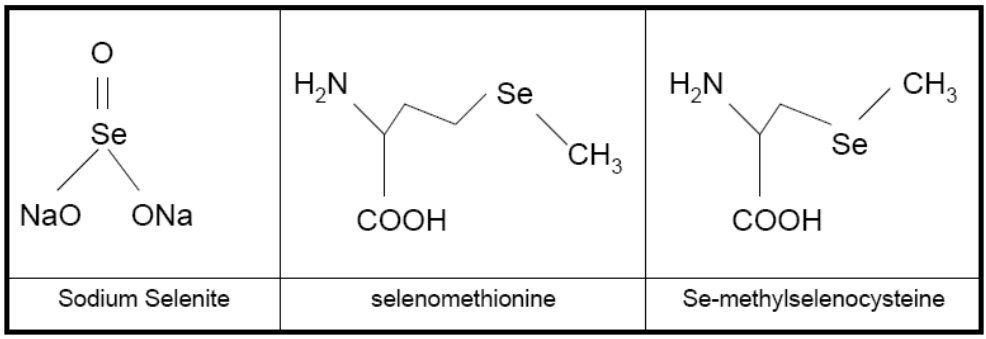

Figure 3. Chemical structure for (a) sodium selenite, (b) selenomethionine and (c) selenocysteine.

Selenoprotein:

25 different selenoproteins containing selenocysteine have so far been observed in human cells and tissues, and some of them have been shown to exert biological functions.[29,30] Four types of Gpx,[31,32,33] three types of thyroid hormone deiodinase,[34,35,36] three types of thioredoxin reductase,[37,38,39] selenophosphate synthetase[40] and selenoprotein P (SeP)[41] were identified as enzymes. Glutathione peroxidase was the first identified mammalian selenoprotein (GPx[1]; EC 1.11.1.9).

Function:
Selenium is of fundamental importance for human health including a proper function of the immune system, prevention of cardiovascular diseases and inflammations. Deficiency of selenium can increase viral infections for example.[42]
Since lack of selenium deprives the cell's ability to synthesize selenoproteins, many health effects of low selenium intake are believed to be caused by the lack of one or more specific selenoproteins. In fact, three selenoproteins, TrxR1, TrxR2 and GPx4, have been shown to be essential in mice knockout experiments.[39] On the other hand, too much selenium in the diet causes toxic effects and leads to selenium poisoning. The recommended daily intake of selenium is 50 and 70 μg/day for women and men, respectively.[43] The threshold between essential and toxic concentrations of this element is rather narrow (the factor is in the range of 10-100).

APOPTOSIS:

Apoptosis or programmed cell death is a normal ongoing phenomenon of cells in living organisms. The ancient Greek word apoptosis (*apo* means from, *ptosis* – falling) used for "dropping off" or "falling of", which refers to the falling of leaves off a tree or petals dropping off flowers was first introduced by kerr et al.[44] in the early 1970s to describe cell death in the absence of any pathological condition. It was based on the following main morphological criteria: cellular shrinkage, plasma condensation, chromatin condensation, DNA fragmentation, cytoplasmic vacuolisation, blebbing. Then, membrane-bound apoptotic bodies which are formed by the nucleus and cytoplasm fragments can be engulfed by phagocytes. This process does not affect neibouring cells. In contrast to apoptosis, necrosis is morphologically characterized by swelling of the cytoplasm and organelles including mitochondia, breakdown of cytoplasmic structures and organelle function and cell rupture. Due to breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular space and can damage surrounding cells often causing inflammation. (Table 1) There are many causes of necrosis including injury,

infection, cancer, infarction, toxins and inflammation. The term necrosis devised from the Greek word used for dead, dead body, dead tissue.

Table 1. Features of apoptosis and necrosis

Characteristics	Apoptosis	Necrosis
Stimuli	Physiological. It can be pathological	Pathological
Cell involved	Single cell	Group of cells
Cell shape	Shrinkage	Cell swelling
Cytoplasm	Late stage swelling	Early stage swelling
Plasma membrane	Intact	Lyses, releasing contents into surroundings
Nucleous	Karyorrexis	Karyolysis
DNA breakdown	Internucleosomal	Randomized
Reaction	Does not trigger inflammatory response	Triggers inflammatory response
Phagocytose	Membrane bound apoptotic bodies phagocytosed	Cellular debris phagocytosed
Level of ATP required	High	Low
Caspases activation	Present	Absent

Regulation of apoptosis:

Apoptotic processes are initiated via extra- or intracellular targets by intrinsic and extrinsic factors.[45] Many different apoptotic signaling pathways have been described.[46, 47] Among them the pro-apoptotic signalling pathway or the extrinsic pathway mediated by specific ligands and surface receptors. The Intrinsic pathway is mediated from mitochondria. (Figure 4) Apoptosis can be divided into three phases: initiation, amplification and execution. In the initiation phase, an interaction between ligands and corresponding death receptors occur. Apoptotic stimuli then activate the caspase cascade, which is called amplification phase. During the excution phase, caspases degrade proteins leading to cell death. In fact, the apoptotic process is very complex since the different pathways may interact with each other.

Extrinsic pathway:

It is also called the receptor mediated pathway due to the death receptors involved in this process. Most death receptors are members of the TNF-receptor (TNFR) super family.[48] So far, at least eight members of the death receptor family have been characterized. (Table 2) The ligands that activate death receptors belong to the TNF gene superfamily[49,50] except the nerve growth factor receptor (NGFR). The CD95 receptor, TRAILR1 or TRAILR2 bind to their respective ligands and forms death inducing signaling complexs (DISCs), which converts procaspase-8 to caspase-8 that in turn activates procaspase-3 and procaspase-9, resulting in apoptosis.[46] (Figure 4) Mitochondria are activated by Fas mediated apoptosis and caspase-8 and release cytochrome c.

Table 2. TNFR Death receptors and their ligands.

Group:	Name of the Death Receptor	Ligands of the death Receptor	Function
Group: 1	CD95 (DR2, APO-1 A and Fas)	CD95L (FasL)	Death inducing signaling complexes (DISCs) are formed at the receptor which plays the central role in apoptotic signal.[51]
	TRAILR1 (DR4, APO-2)	TRAIL (Apo2L)	
	TRAILR2 (DR5, KILLEER, TRICK2)	TRAIL (Apo2L)	
Group: 2	TNFR1 (DR1, CD120a, p55)	TNF and Lymphotoxin alpha	Promotes both apoptotic and survival signals via different set of molecules.
	DR3 (APO-3, LARD, TRAMP, WSL1)	Apo3L (TWEAK)	
	DR6 (Ectodisplasin A receptor EDAR)	May belong to the TNFR family	
	Nerve growth factor receptor (NGFR)	Ligand does not belong to the TNF gene superfamily	
	CAR1	Unknown	

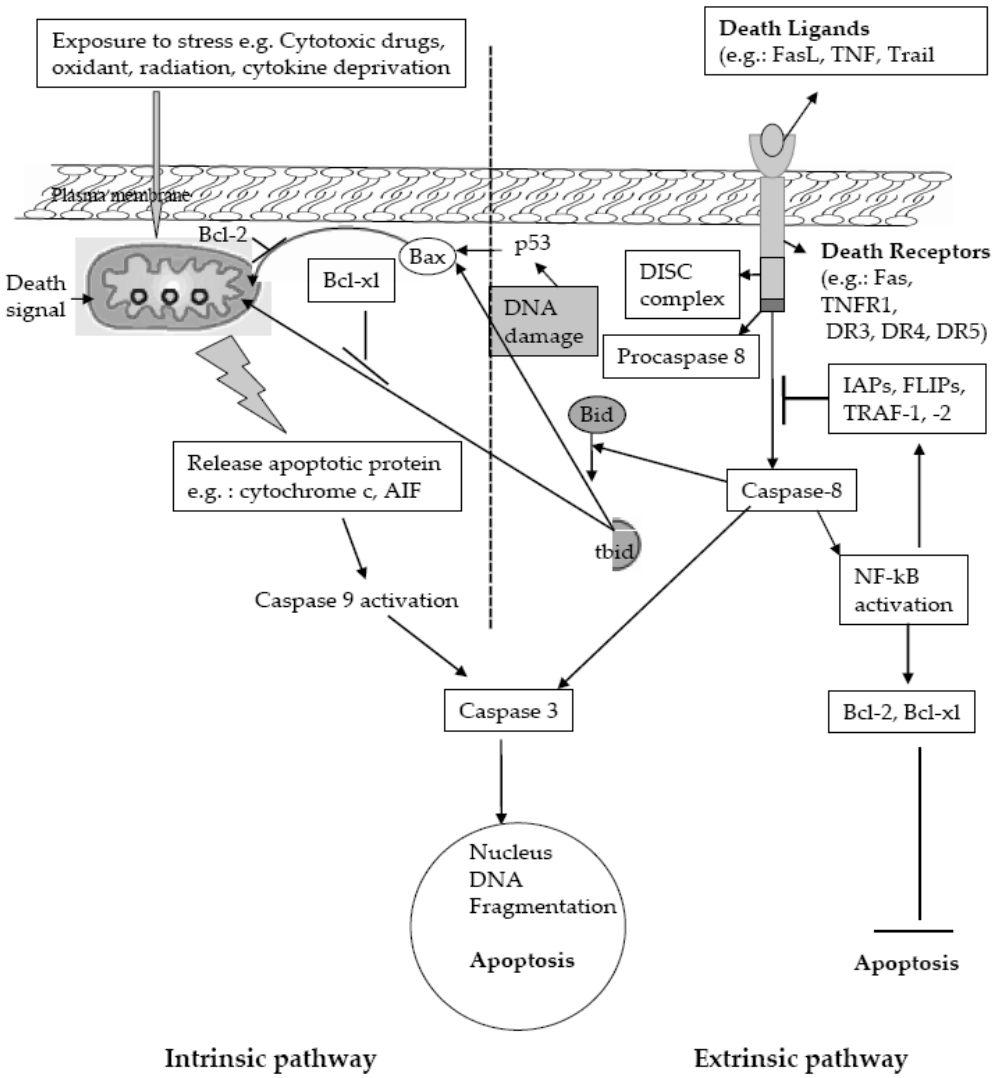

Figure 4. Schematic representation of extrinsic and intrinsic apoptotic pathways.

Intrinsic pathway:
Mitochondria are called the cell´s power house and play a main role for the regulation of apoptosis in the intrinsic pathway. The intrinsic pathway can be divided into pre-mitochondrial and post-mitochondrial regulation.
In the pre-mitochondrial pathway, the mitochondria is activated by different signaling molecules including the Bcl-2 protein family and the p53 protein and it has both anti-apoptotic and pro-apoptotic factors. The anti-apoptotic factor Bcl-2 prevent apoptosis by inhibiting mitochondrial permeability transition (MPT).[52] Pro-apoptotic factors are for example Bax and the tumor suppressor p53. Bax enhance the release of apoptotic molecules, i.e. cytochrome c,[53] whereas p53 induce apoptosis direct and indirect by activating the transcription of pro-apoptotic genes and form a complex with the anti-apoptotic proteins Bcl-2 and Bcl-Xl.[54]

In post-mitochondrial regulation, apoptosis is regulated by many factors including pro-apoptotic factors, cytochrome c, apoptosis-inducing factor (AIF) and endonucleases. Cytochrome c is normally localised in the intermembrane space and it is translocated to the cytosol. There it forms apoptosome complexes by interacting with Apaf-1 and procaspase-9 to initiate caspase activation which lead to proteolysis and cell death.[46,55]
In contrast to cytochrome c, AIF can induce caspase independent cell death.[56]

Redox regulation of apoptosis:

Redox regulation plays an important role in the regulation of apoptosis. Trx and GSH system activate transcription factors and several components of the apoptosis pathways. Excess ROS can be dangerous to cells but ROS also act as a redox signaling molecule like second messengers in several transduction pathways.[57] ROS regulate cellular growth and death through different pathways.

Mitogen activated protein kinase (MAPK) family is a large family of serine/threonine kinases, which has three subfamilies ERK (extracellular signal-regulated kinasea), JNK (Junc N-terminal kinase) and P38.
ERK has links to cellular proliferation, while JNK and p38 pathways are associated with stress responses. JNK and p38 subfamilies are activated by stressfull agents such as ROS, heat, radiation, shock[58] and referred to as stress activated protein kinases (SAPK). Mitogen activated protein kinase kinases (MAPKK) and mitogen activated protein kinase kinase kinases (MAPKKs) are essential for JNK and p38 pathways.

Apoptosis signal-regulating kinase 1 (ASK-1) is a most important member of MAKKs for the JNK and p38 pathways. ASK-1 is kept inactive in the apoptotic signalling pathway under normal conditions by having reduced Trx structurally associated to itself.[59] But under oxidative conditions, Trx becomes oxidized and dissociates from ASK-1, which leads to activation of the downstream targets of ASK-1, JNK and p38 involved in apoptotic pathways. It also may lead to both caspase-dependent and caspase-independent apoptosis. However, redox regulation of apoptotic pathways are very complex and both JNK and p38 have pro-apoptotic and anti-apoptotic effects within the cell.[58, 60,61, 62]

Transcription factors in Redox-regulation:

Transcription factors are proteins composed of two essential functional regions: a DNA binding domain and an activator domain. Transcription factors can be activated or deactivated by others protein. Without transcription factors, formation of new mRNA from DNA is impossible. The transcription factors can be activated by physiological, therapeutical and pathological stimuli. Most important transcription factors are Rel/NF-κB, p53 and AP-1, further described below.

NF-*k*B

NF–κB is a ubiquitous redox-regulated transcription factor that is characterized by the presence of a conserved 300-amino acid Rel homology domain, which is responsible for dimerization, interaction with inhibitor of κB (IκBs) and binding to DNA. It was first identified in 1986 as a nuclear factor necessary for immunoglobulin kappa light chain transcription in B cells.[63] The transcription factor NF-κB consists of homo- or heterodimers. The NF-κB protein can be divided into two groups according to the presense or absence of potent transactivation domains (TDs), which are essential for transactivation activity. NFκB is retained in the cytoplasm in an inactive form bound to IkBs, which prevents the NF-κB:IκB complex from translocating to the nucleus.[64,65,66]

NF-κB binds to DNA as a dimer that can be composed of the subunits RelA/p65, p50, p52, c-Rel and Rel-B. The p65 subunit is important for gene transcription.[67] NF-κB controls the expression of genes involved in mediating innate and adaptive immune responses. In most cells, NF-κB mediates cell survival signals, but under certain conditions, it may induce apoptosis. NF-κB activation also functions in the antiviral response through interferon gene regulation. Inappropriate regulation of NF-κB contributes to a wide range of human disorders, including cancers, neurodegenerative disease, arthritis, asthma, inflammatory bowel disease and viral infection. So far five mammalian NF-κB family members have been identified: (Table 3)

Table 3. NF-κB family members:

NF-κB subunit	Size	Special characters
NF-κB1 /P50	50 kDa	Synthesized precursor IκB protein p105. It does not posses TD.
NF-κB2 /P52	52 kDa	Synthesized precursor IκB protein p100. It does not posses TD.
Rel A /P65	65 kDa	The C-terminal region of RelA contains a putative leucine zipper domain and a transactivation domain that is important for the NF-κB-mediated gene transactivation.
Rel B /P68	68 kDa	It does not possess PKA phosphorylation site but an additional N-terminal affects its transcriptional activity.
c-Rel B		unknown

Other Rel members are Dif, Dorsal and Relish (Drosophila), v-Rel (chicken oncogen), c-Rel, p52 and Rel-B.
The production of cytokines such as IL-6, IL-1β or TNF is dependent upon the activity of NF-κB transcription factors.[68] NF-κB is rapidly activated in response to proinflammatory stimuli, infections, physical and chemical stressors.

P53:
It is also known as tumor suppressor protein 53 (TP53) or "Guardian of the Genome". It is an essential transcription factor for regulation of cell cycle control and apoptosis including DNA repair and cell-survival which is redox-regulated.[69] The p53 protein is activated in response to oxidative stress, radiation, chemical agents, and cytokines. Activation of p53 induces p21, which inhibits the formation of Cdk2 and complex required for the cell cycle and thereby leads to cell cycle arrest. In addition, p53 repair multiple types of DNA damages such as nucleotide excision repair, base excision repair and correction of double strand breaks.[70] Since p53 have multiple

functions, loss of p53 functions in a cell is dangerous. Deletions or mutations of the p53 gene is very common in cancer.

AP-1 :

AP-1 is a key transcription factor, which is involved in cellular proliferation, transformation and death.[71] AP-1 belongs to the basic leucine zipper (BZIP) protein group composed of Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra1 and Fra2) and activating transcription factor (ATF) protein family members.[72,73]

Protein kinase C (PKC):

PKC is a family of protein kinase with many different isozyme members that can modify the activity of other proteins in a cell. In mammalian tissue, eleven isoforms, divided into three classes, have been identified.[74,75] PKCs can not activate other protein without phospholipids, which are not able to activate the protein by themselves. The conventional PKC isozymes are activated by Ca^{2+}, whereas the novel isozymes are Ca^{2+} independent phorbol ester receptor/kinases. The atypical PKCs are also Ca^{2+} independent kinases but do not bind phorbol esters or diacylglycerol.[76,77] Thioredoxin (Trx) inhibits PKCs autophosphorylation and PKC-mediated phosphorylation of histones with an IC_{50} of 20 ng/ml (equivalent to 1.6 nM) in *vitro*.[78]

Redox-regulated Cytokines:

The word cytokine derived from cyto which means cell and kinesis meaning movement. These are low molecular weight proteins released by cells (both hemopoietic and non-hemopoietic) involved in communication between cells in order to regulate the immune system. Cytokines also influence many types of cells not belonging to the immune system. Cytokine is a general name, others names are lymphokine, monokine, chemokine and interleukin. More than 100 different cytokines have been identified. Cytokines may have multiple functions and play an important role in immune regulation as well as in neuro-immune-endocrine modulation.[79] They act at very low concentrations and each cytokine binds

to a specific cell-surface receptor. Examples of receptors and their corresponding cytokine family are hematopoietin family, interferon family, TNF family and chemokine family.
Their actions are grouped as i) autocrine; ii) paracrine; iii) endocrine. All cytokines are classified into three categories,[80] summarized in Table 4.

Table 4: Classification and function of cytokines

Name of the categories	Example of cytokines	Function
Mediators and regulators of innate immunity	TNF, IL-1, Type 1 interferons, IL-6, IL-10, IL-12, IL-15 and chemokines	Induce early inflammatory reactions to infectious agents.
Mediators and regulators of adaptive immunity	IL-2, IL-4, interferon γ, IL-5, lymphotoxin, TGF-β, IL-13, IL-16, IL-17 and migration inhibiting factor MIF).	Act primarily on other lymphocyte populations, regulate their growth and differentiation
Stimulators of hemopoiesis	Stem cell factor (also called steel factor or c-kit ligand), IL-3, IL-7, granulocyte-monocyte colony stimulating factor, IL-9 and IL-11.	Act as growth and differentiation factors of leukocyte precursors of various lineages.

From the view of inflammation, cytokines are divided into two groups: a) pro-inflammatory cytokines, which are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. b) Anti-inflammatory cytokines belong to the T cell-derived cytokines and are involved in the down-regulation of inflammatory reactions. Many studies suggest that cytokines induce apoptosis in cancer cells[81,82,83] and this activity has been shown in a wide range of cancer types including leukemia, breast, bladder, melanoma, ovarian and prostate.
TNF is a very important pro-inflammatory cytokine which is involved in many pathologies including chronic heart failure, B-CLL, cancer, inflammatory bowel disease, multiple sclerosis. The importance of TNF and TNFR superfamily will be discussed below.

Tumor necrosis factor (TNF):
TNF was first discovered as a serum factor produced in mice treated with LPS in 1975[84] and it was called TNF because it showed antitumor effects both *in vitro* and *in vivo*. TNF is produced by many different cells including macrophages, monocytes, neutrophils, lymphocytes, and fibroblasts[85] in response to environmental challenges including inflammation, cancer, and infection. TNF may elicit its effects by binding to TNFR1 which is found on most cells in the body and TNFR2, which is primarily expressed on hemopoietic cells. Most biological effects of TNF are mediated through its binding to TNFR1 which leads to recruitment an adaptor protein that activates multiple signaling pathways.[86,87] TNF has been shown to regulate cell death, proliferation, differentiation and survival.[88] TNF is involved in redox-regulation, which is apparent from a prompt ROS induction upon TNFR-TNF ligand binding.[89]

TNF in inflammation including chronic heart failure (CHF) and cancer:

TNF is the most prominent inflammatory mediator and plays a central role in inflammatory reaction of the innate immune system including cytokine production, activation, immune-cell proliferation, expression of adhesion molecules and induction of inflammatory processes.[90,91,92,93,94,95] Large amounts of TNF is produced under severe inflammation and infection. TNF then enters the blood stream causing fever, hypotension and shock[96] leading to damage of cells or tissues.[97] Overexpression of TNF can lead to inflammatory disease including inflammatory bowel disease (IBD), Crohn´s disease (CD), ulcerative colitis, rheumatoid arthritis (RA), and multiple sclerosis (MS)[98,99,100] due to an imbalance between inflammatory and anti-inflammatory cytokine production**.** In CHF, circulating levels of TNF are elevated and increased levels are found as the patient´s CHF worsen.[101] We found support of these observations in paper II. Normal myocardium expresses TNFR1 and TNFR2, but it does not contain TNF. In heart disease, TNF receptors are downregulated and expression of TNF increased.[102] It has been shown, that TNF provides a stimulus for growth of myocytes,[103] which leads to hypertrophy and protects myocardial cells from hypoxia.[104]
TNF is not normally detected in plasma/serum, but can be detected in cancer patients including B-cell chronic lymphocytic leukemia (B-CLL),[105] pancreatic cancer,[106] ovarian cancer,[107] breast cancer,[108] multiple myeloma[109] and non-Hodgkin´s lymphoma.[109] The TNF level correlates with disease stage**.** TNF activates NF-κB and AP-1 transcription pathway.

Thioredoxin

Thioredoxin (Trx) is a 12kDa small redox-active protein found in all living organisms. Trx plays multifunctional roles in cell proliferation, cancer,[110] and different diseases including inflammation.[111]
Trx serves as a general protein disulfide oxidoreductase, having a highly conserved active site sequence Cys-Gly-Pro-Cys, which facilitates protein disulfide-dithiol exchange. Trx was originally identified in 1964, as an electron donor for ribonucleotide reductase in *Escherichia coli* and was source to be essential for DNA synthesis.[112] The crystal structure of Trx was first determined in 1975[113] (Figure 5 adapted from Holmgren, 1995)[114] and two forms have been found: i) oxidized thioredoxin (Trx-S_2) with a disulfide and ii) reduced thioredoxin [Trx-$(SH)_2$] with a dithiol.[115] Trx can also function as a free radical scavenger.[116] Through these activities Trx is able to regulate the redox state not only in protein targets, but also in the entire cellular environment. In addition, Trx has been shown to have both direct and indirect antioxidant effects.[117]

Biological roles of Trx:

As mentioned, Trx have multifunctional biological roles both intracellularly and extracellularly, which may be either dependent or independent of its redox activity. These important roles are:
a) Protection of cells against oxidative stress. b) Effect on cell proliferation. c) Protection against apoptosis. d) Regulation of chemoattractant activity. e) Transcription factor regulation. Each of these roles are discussed in detail below.

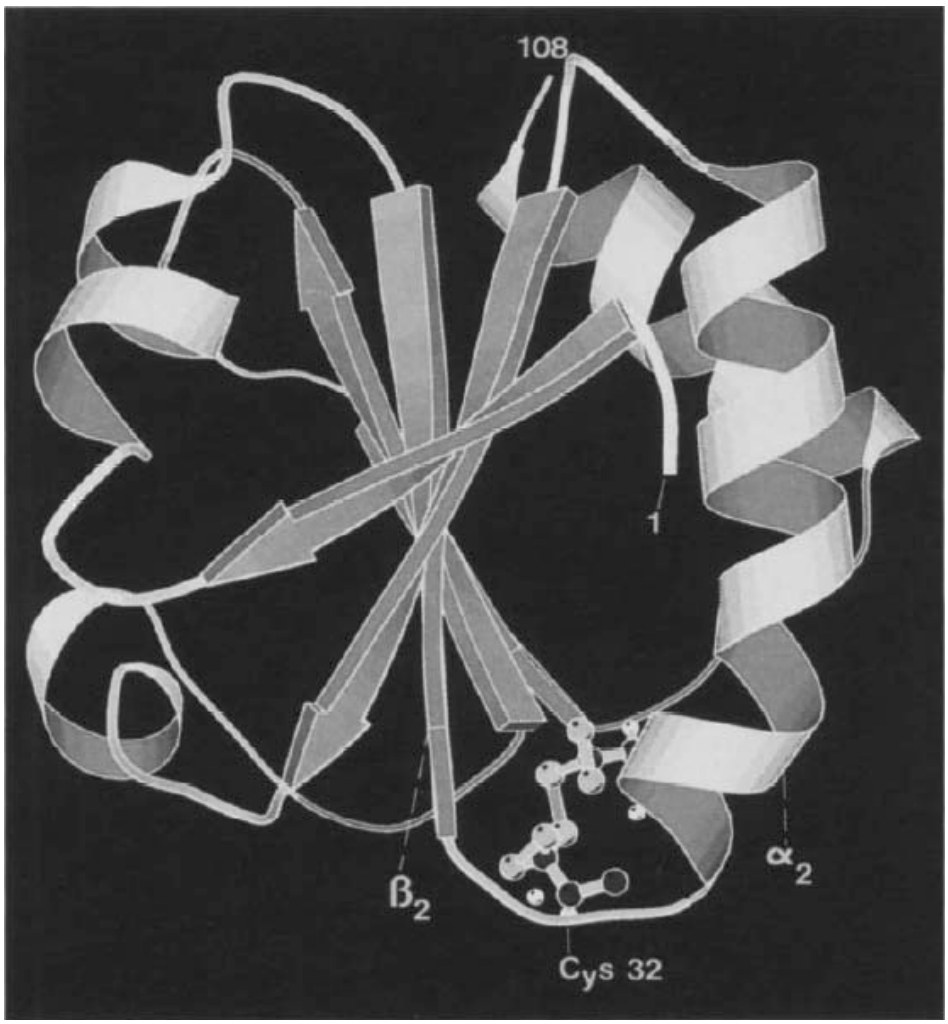

Figure 5. Folding of thioredoxin. The secondary structure of thioredoxin from coordinates for *E. coli* Trx-S_2 obtained by NMR in solution.[118] It consists of five-stranded β–strands surrounded by four alpha helices. Note that the redox-active disulfide in the active site (Cys32-Cys35) is located on a protrusion between the strand β_2 and the helix α2. Only the sulfur of Cys32 is exposed to solvent. Adapted from Holmgren, 1995.[114]

Trx protect cells against oxidative stress:

Protection from oxidative stress is the main function of thioredoxin. Trx expression is upregulated by different oxidative stress inducers including UV exposure and H_2O_2.[119] Trx protects against H_2O_2 and TNF-mediated cytotoxicity, a pathway in which TNF receptor-binding generates ROS. In excess amounts, ROS is cytotoxic and activates the apoptosis signal regulating kinase 1 (ASK-1) in the cytoplasm by releasing Trx that is structurally complexed to ASK-1.[120] Trx is thus an exquisite redox-sensor. Trx has also direct antioxidant activity and reduces ROS through an interaction with the redox-center of Trx (-Cys-Gly-Pro-Cys-).[121,122]
In addition to its intracellular functions, Trx is released under physiological conditions of oxidative stress caused by a variety of stimuli such as mitogens and inflammatory signals,[123, 124] viral infections, such as HIV[125] and severe burns.[126]

Effect on cell proliferation and growth:

In 1985 Teshigawara suggested that adult T-cell leukemia derived factor (Trx) promotes cell growth[127] and reduced Trx was found to promote growth of a human T cell leukemia virus infected cell line and increased the expression of the IL-2 receptor. Our group previously reported growth stimulatory pathways of Trx and cytokines [128-130] and cytokine receptors (IL-2R).[131] In contrast to intracellular Trx, extracellular Trx is generally considered to be present in an oxidized form, devoid of protein disulfide reductase activity, but having gained the function of a true chemokine.[125] Previous reports show that circulating Trx suppresses lipopolysaccharide (LPS)-induced neutrophil chemotaxis,[132] and injection of human Trx has been demonstrated to reduce ischemic reperfusion injury, mainly through reduced leukocyte extravasation.[133]

Inhibition of apoptosis:

Trx has gene regulatory activity of several transcription factors such as glucocorticoid receptor[134] and NFκ-B via thiol-disulfide cystein control of DNA binding. Trx also controls apoptotic or hypertrophic pathways decision in a fascinating redox-sensitive "on-off" mechanism.[135,136]

Regulation of chemoattractant activity:

Trx and in particular the truncated form of Trx, acts as a chemoattractant for neutrophils, monocytes and T cells in culture.[125] Trx does not induce intracellular calcium and the process is G protein independent. In contrast to these result, lipopolysaccharide stimulated neutrophil migration in a murine air pouch model decreased by increased Trx.[137]

Protein disulfide isomerase (PDI):

PDI, a multifunctional 55kDa redox active protein was first purified from rat liver microsomes as a thiol disulfide exchange enzyme.[138] In 1985, the first full-length cDNA sequence on PDI, which encode for 508 amino acids, came from rat liver and revealed that rat liver enzyme contains two domains that are homologous to thioredoxin.[139]

The enzyme is localized primarily in the endoplasmic reticulum of eukaryotic cells (yeast, plants, mammals) and nuclear localization has been reported.[140] PDI has also been found to be secreted from endothelial cells,[141] hepatocytes,[142] pancreatic exocrine cells[143] and platelets.[144] It has also been found on the cell surface of a variety of different cell types including retina in chicken embryo[145], platelet plasma membrane,[146] B lymphocytes,[147] and B-CLL cells.[148]

Function:

It is generally considered that PDI is important as a physiological catalyst for the formation of native disulfide bonds.[149] It is capable of catalyzing both oxidation and reduction of disulfides under physiological condition. PDI has a chaperone activity and can assists folding of proteins with no disulfides[150,151] such as D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH),[152] rhodanesse[153] and disulfide-containing proteins, such as lysozyme[154]. Puig and Gilbert (1994) found chaperone and anti-chaperone activity of PDI in the refolding of lysozyme.[155]

PDI is essential for the assembly of some multifunctional proteins such as prolyl-4-hydroxylase[156] and microsomal triglyceride transfer protein complex.[157] Yamauchi et al. (1987) reported that tri-iodothyronine binding protein is identical to PDI.[158] Since tri-iodothyronine is a nuclear receptor for induction and trancriptionon of genes, the physiological significance of this finding is unclear. Primm and Gilbert (2001) reported that PDI binds estradiol and tri-iodotyronine via a distinct hormone binding site and suggested that PDI act as a hormone reservoir or mediates hormone-receptor binding.[159] Cell surface PDI may play a role for transfer of nitric oxide into cytosol from extracellular proteins by an unknown mechanism.[160] Platelet surface PDI may have a role in platelet activation via its oxido-reductase activity.[161]

PDI family:

The PDI family consist of PDI and PDI like protein. The most known PDI family member are PDI, PDIp, ERp57, ERp72, p5, PDlr, ERp28[162,163] and TMX[164]. In addition to these PDI family members, several new PDI members have been reported including ERp44,[165] ERp46,[166] Erp18,[167] ERdj5,[168] thioredoxin-related transmembrane protein 2 (TMX2),[169] and PDILT.[170] Schematic overview of the human PDI family is shown in figure 6.[171]

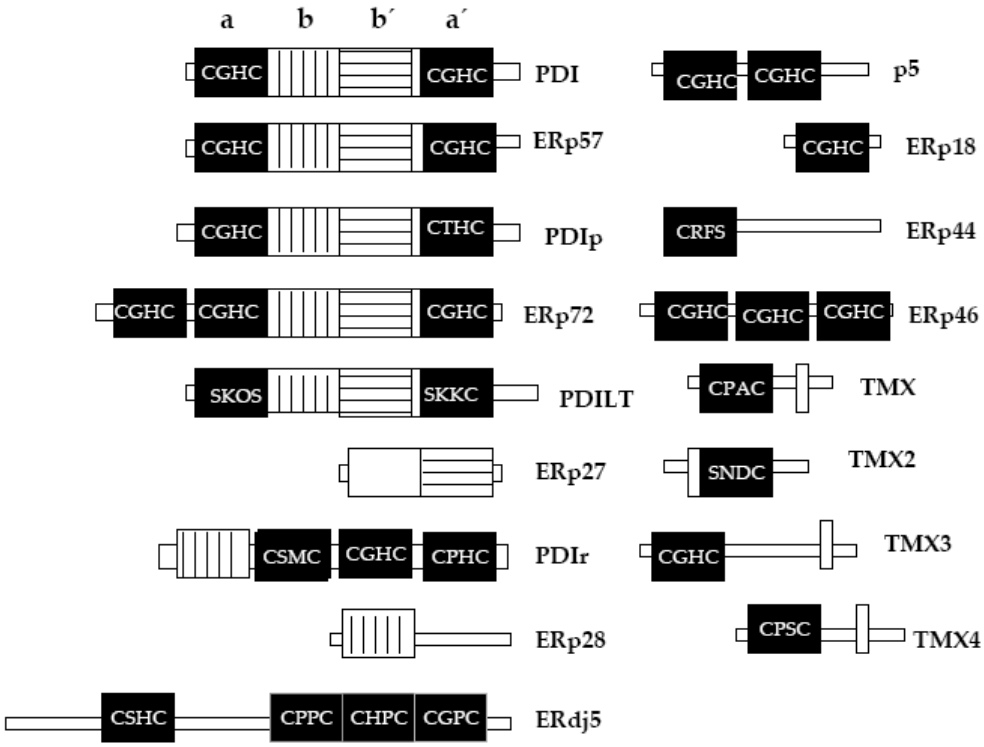

Figure 6. Schematic overview of the human protein disulfide isomerase family. Trx-like domains are represented by rectangles with the active-site sequence added for catalytic domains (black). Modified from Ellgaard and Ruddock 2005.[171]

Chronic Lymphocytic Leukemia (CLL):

B-cell chronic lymphocytic leukemia (B-CLL) is the most common leukemia of adults in the Western countries, accounting for 30% of all leukemia cases. About 400-500 new cases are found each year in Sweden alone, and most patients are over 50 years of age at the time of diagnosis. Many CLL patients survive up to 30 years from diagnosis with or without treatment due to slow progression. Many cases of CLL are first diagnosed by routine blood test.
CLL includes both cases with indolent disease and a more aggressive variant. CLL patient may have signs and symptom such as enlarged lymph nodes, enlarged liver and spleen, fatigue, bone pain, excessive sweating, loss of appetite, weight loss, flank pain, and generalized itching. Abnormal bruising is a common symptom of CLL, but often does not appear until late in the illness. CLL is approximately two times more common in men than in women. Currently, there is no curative therapy for CLL but new research is bringing new approaches to managing the disease for improved treatment.

Two major clinical staging systems (Rai and Binet) were developed based on the progress of the tumor to estimate prognosis of CLL.[172,173,174] Both systems were indicated only when progression or disease related symptom supervene. Therefore, continuous efforts have been made to identify additional prognostic factors, which may aid to better understanding of B-CLL and prognosis of the disease. In B-CLL, chromosomal abnormalities are observed in about 50 percent of cases.[175,176,177,178,179, 180] In 1999 Hamblin et al and Damle et al showed that B-CLL could dividable into two subsets from the presence or absence of mutations in the immunoglobulin (heavy chain (IgVH).[181,182] It was proven that patient with the mutated B-CLL subset had significantly longer survival. The absence of IgVH mutations also related with a high risk of CLL progression.[181,183,184]

The diagnostic hallmark of B-CLL is an accumulation of monoclonal $CD5^+$ positive B cells resting in the G0/G1 stage of the cell cycle, that express a limited IgVH-gene repertoire. Typical B cell surface antigens are present but only low amounts surface IgM/IgD.[185,153] The proto-oncogene bcl-2 is upregulated 1.5 to 25 fold in most cases of B-CLL.[186,187] The Bcl-2 protein overexpression explains the successive expansion of the malignant clone despite a minimal proliferating cell fraction. Bcl-2 expression is enhanced by cytokines such as IL-4, IFN-α, IFN–γ, bFGF and CD6-ligation.[188,189,190, 191,192] In contrast, glucocorticoids, IgM-ligation, IL-10, or growth factor withdrawal leads to bcl-2 downregulation and *bax* upregulation.[192,193] High Bcl-2/Bax ratios were found to protect against apoptosis.[192] It is not known by which mechanism B-CLL cells proliferate, but physiological stimulation through membrane receptor-ligation, and cytokine ligand-receptor interactions will induce mitogenic responses, although often weak.[194,194,195,196,197,198, 199] One of the cytokines that contributed to S-phase entry and mitosis in synergy with IL-2, IL-4, TNF, and CD40-ligation was thioredoxin (Trx).[196] Our group found that redox active protein Trx prolongs survival of B-type chronic lymphocytic leukemia cells.[200] Studies on PDI shows that it is highly expressed on B-lymphocytes, particularly B-CLL cells.[148]

Chronic Heart Failure:

Chronic Heart failure (CHF) is a complex clinical syndrome which has been defined in many different ways. CHF is a multisystem disorder, which affects many systems including renal, neuroendocrine, musculoskeletal, and immune system. Clinically, CHF is characterized by symptoms such as exertional breathlessness and fatigue, signs of fluid retention as well as signs associated with the underlying cardiac disorder. These symptoms can result from any structural or functional cardiac or non-cardiac disorder. Neurohormonal or inflammatory mechanisms may play a main role in the disease process.[201] CHF can also result from an imbalance between ROS and anti-oxidative cellular defense mechanism.[202] CHF is associated with excess ROS[203,204] and ROS is involved in the progress of the disease.[204,205,206] Ruffolo and Feuerstein (1998) proposed that excess ROS may contribute to the activation of transcription factors which lead to apoptosis.[207] TNF, which is produced by inflammatory cells (e.g. monocytes, neutrophils), and ROS are able to induce apoptosis in myocytes and endothelial cells,[208] thus playing a important role in the progression of the disease.[209] It has been reported that intake of antioxidants decrease the risk of coronary heart disease.[210,211,212]

Aims of the thesis:

The overall aim of this thesis was to investigate the importance of redox active proteins and cytokines in inflammation and cancer.
Specific aims:

1) The role of Trx, TrxR, and selenium in carcinogenesis and in resistant cancer cells.
2) The potential role of Trx as a key regulator in cellular redox balance, in the pathogenesis of cardiac dysfunction; its relationship to stress response parameters.
3) The importance of Trx in cancer cells and the redox regulation of TNF and its receptors TNFR1 and TNFR2.
4) Whether unmutated CLL (U-CLL) responses to PKC and ROS pathways were different from mutated CLL (M-CLL) responses.

Results:

Paper I:

Selenite-induced apoptosis in doxorubicin-resistant cells and effects on the thioredoxin system

In this study, we focused on the role of Trx, TrxR, and selenium in the carcinogenesis and resistance of mesothelioma cancer cells.
Selenite-induced apoptosis was determined in a concentration and time dependent manner by TUNEL-assay, morphological investigations and FACS analysis. We observed that after continuous incubation of 10 μM selenite the doxorubicin sensitive cells showed maximum apoptosis at day 4. The doxorubicin-resistant cells, however, showed a maximum of apoptosis already at day 2. Increasing concentrations of selenite did not significantly increase apoptosis but necrosis at the highest concentration. Selenite induced-apoptosis in the drug resistant cells seemed to be caspase-3-independent. We observed that selenite induced apoptosis in a significantly larger portion of the doxorubicin-resistant cells compared to the doxorubicin-sensitiv cells.
TrxR activity and amount was determined by ELISA technique. After 2 days of incubation, basal activity of TrxR was similar in both cell lines but basal activity was higher in the doxorubicin-resistant cells after 4 day incubation. Results showed that the maximum increase of TrxR was achieved already at 1 μM of selenite.
We also measured the level of Trx and truncated Trx in tumor cell extracts by ELISA techniques. Selenite treatment did not alter the level of Trx in these two cell lines, but the level of tTrx was higher in drug resistant cells.

Paper II

Elevated circulating levels of thioredoxin and stress in chronic heart failure.

Twenty-seven male patients with CHF and 29 healthy controls were studied in paper II. Our study population was restricted to males in order to obtain a homogenous population, reducing possible gender specific hormonal influences. The clinical stage was assessed according to the NYHA functional class. We investigated the circulating Trx and TrxR redox-proteins in relation to biochemical stress and inflammatory markers. We measured Trx, TrxR, IL-6, P-selectin by ELISA techniques and lipid peroxides were determined by the TBARS assay (detecting TBA-malondialdehyde complexes) that are generated upon oxidative stress. Salivary cortisol was analyzed by a biotin-streptavidine immunoassay. For all assays used in the study, each sample was tested in triplicates in three separate experiments, for mean value determination, except for saliva samples, which were tested in triplicates once. We also calculated intra- and inter-assay coefficients of variation in the immunoassays. We found that plasma level of Trx was significantly higher than healthy control group ($p = <0.0001$). The Trx levels increased in proportion to the severity of the disease. We also observed that Trx was significantly associated with lipid peroxides, salivary cortisol and serum creatinine. Interestingly, we found that Trx-reductase significantly correlated with TNF and IL-6 and TNF correlated with IL-6.

Paper III

Membrane protein disulfide isomerase regulates TNF-receptors 1 and 2 via direct molecular interaction: Redox-control of TNF autocrine loop in CLL.

PDI is a multifunctional cytoplasmic enzyme, which is known to catalyze the formation of disulfide bonds. The mechanisms behind the observations that Trx is responsible for the growth and survival of B-CLL is not clear, we hypothesized that Trx and PDI are responsible for a very rapid and fascinating thiol-disulfide modulation of cystein-rich surface membrane receptors. All members of the TNF-superfamily contain multiple extracellular cystein-rich domains. In addition, the membrane receptors CD5 and CD6 belong to the cystein-rich scavenger receptor family. Based on these observations, we asked whether the redox active proteins Trx and PDI are involved in the delicate control of the TNF autocrine loop? If yes, how? We hypothesized that PDI regulates signalling via the TNFR1 and TNFR2 and thereby control TNF autocrine release. For this reason, we intervened with the redox-pathways in B-CLL lymphocytes by blocking the activity of Trx and PDI by specific antibodies or inhibitors or by generating oxidative stress.

Our experimental techniques were: i) We measured cell viability by the trypan blue dye exclusion. ii) TNF was analyzed in the presence or absence of inhibitors of the redox-active proteins Trx and PDI by enzyme immunoassays. iii) TNF, TNFR, Trx and PDI expression determined by FACS using specific antibodies in FACS analysis. iv) Molecular interaction of surface membrane TNFR (TNFR1 and TNFR2) and PDI was studied by receptor membrane clustering/co-capping in deconvolution microscopy using multicolor immunofluorescence.

Flow cytometry analysis showed PDI, TNFR1 and TNFR2 expression on B-CLL surface. We found that TNFR1 and TNFR2 were physically associated with PDI, as shown by co-immunoprecipitation and co-capping, revealing a molecular interaction at the outer surface membrane. The autocrine TNF release was blocked by different inhibitors of PDI such as bacitracin and pentoxifylline. This was verified by two anti-PDI antibodies.

We also found that oxidative stress generated by sodium selenite or diamide inhibited TNF release from B-CLL cell.

Paper IV

Increased thioredoxin and TNF expression in unmutated CLL compared with mutated CLL cells after protein kinase C activation

In this study, we included 16 patients (10 cases with unmutated IGHV gene and 6 cases with mutated IGHV gene).
We investigated whether U-CLL response to the inositiol phospholipid pathway by phorbol-12-myristate 13-acetate and the calcium ionophore ionomycin, were different from M-CLL responses. Proliferation responses were measured by thymidine incorporation. We also investigated the expression and/or secretion of the potential tumor promoting factors Trx, TNF and PDI in U-CLL and M-CLL. Viability was analyzed by a method from Guava Technologies. We also used annexin V-FITC to determine apoptosis of tumor cells. Furthermore, we determined the expression of Trx, PDI and TNF by FACS analysis. We observed that PMA/ionomycin induced proliferation both in U-CLL and M-CLL but there was no significantly difference in proliferation between U-CLL and M-CLL. We also found that the frequency of apoptotic cells was not significantly changed in U-CLL nor in M-CLL after PMA/ionophore stimulation. However, the analyzes clearly showed that the increase in Trx and mTNF expression after stimulation was significantly higher in U-CLL compared with M-CLL. PDI expression was at the same level both in U-CLL or M-CLL after stimulation.

Summary and Conclusions:

- Our results demonstrate pronounced selective selenium-mediated apoptosis in therapy resistant tumor cells and suggest that redox regulation through the thioredoxin system is an important target for cancer therapy. (**Paper I**)
- We also studied the potential role of Trx, which is a cytoprotective protein and key regulator of cellular redox balance, in an inflammatory pathological situation, namely that of chronic heart failure, which is a disease characterized by cardiac dysfunctions with several overexpressed pro-inflammatory cytokines in late progression stages. We also studied the relationship of the Trx-system to stress response parameters. Trx was strikingly elevated in heart failure cases compared with controls, signifying an adaptive stress response that is higher the more severe the disease *e.g*. increased NYHA functional class and oxidative stress. These observations cast new light on the importance of oxidative stress and stress adaptation in CHF and offer a rationale for intervention, aiming at reinforcing the beneficial cytoprotective effects of the selenium-dependent Trx-system. (**Paper II**)
- We carried out detailed analysis of the importance of Trx in cancer cells (B-CLL) and the redox regulation of TNF and its receptors TNFR1 and TNFR2. TNF autocrine release was redox modulated and the TNF receptors interacted at the cell surface membrane with the redox-active PDI, which exerted a stringent redox-control of the TNFR signaling. These results indicate that thiol-disulfide modulation is important in TNF receptor control thus in regulation of survival growth and apoptosis in B-CLL cells. (**Paper III**)
- Our result suggest that U-CLL is more prone to produce the potential tumor promoting factors Trx and TNF compared with M-CLL, which partly could explain the more aggressive behavior of U-CLL. (**Paper IV**)

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